

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68	A1	(11) International Publication Number: WO 00/05409 (43) International Publication Date: 3 February 2000 (03.02.00)
<p>(21) International Application Number: PCT/GB99/02359</p> <p>(22) International Filing Date: 21 July 1999 (21.07.99)</p> <p>(30) Priority Data: 9815799.3 21 July 1998 (21.07.98) GB</p> <p>(71) Applicant (for all designated States except US): PHARMA-GENE LABORATORIES LTD. [GB/GB]; 2A Orchard Road, Royston, Herts SG8 5HD (GB).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): CAREY, Janet, Elizabeth [GB/GB]; Pharmagene Laboratories Ltd., 2A Orchard Road, Royston, Herts SG8 5HD (GB).</p> <p>(74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p>	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: QUANTITATIVE ANALYSIS OF GENE EXPRESSION USING PCR</p> <p>(57) Abstract</p> <p>The invention provides an improved method for quantitation of two target genes in a simultaneous PCR, without the need to determine limiting primer concentrations for both targets. In particular, the invention provides a method of quantitation of expression of a first and a second target protein-encoding nucleic acids using a PCR based assay in which amplification of nucleic acid is detected by release of a reporter fluorescent signal which is measured as a normalised reporter value ("deltaRn").</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

QUANTITATIVE ANALYSIS OF GENE EXPRESSION USING PCR

Field of the Invention

The present invention relates to a method of quantitative
5 analysis of gene expression that is particularly suited for use
in automated assay systems involving fluorescent dyes.

Background to the Invention

Of the systems currently available for automated assay to
determine mRNA copy number of target gene transcripts the
10 Perkin Elmer Applied Biosystems® ABI PRISM® 7700 sequence
detection system and associated TaqMan® probes are one of the
most efficient and effective and most widely used.

The Perkin Elmer System is a PCR (Polymerase Chain Reaction)
based system in which a probe labelled with a fluorescent
15 reporter molecule (typically 6-FAM, JOE or VIC - see below) and
a quencher dye (e.g. TAMRA - see below) is provided which
hybridises to the target nucleic acid between the site of
hybridisation of the forward and reverse PCR primers. While
intact, any fluorescence emitted from the probe's reporter
20 molecule is absorbed by the quencher.

During the primer extension phase of the PCR, the Taq
polymerase displaces the reporter probe from the strand of DNA
being amplified, and in doing so the 5' → 3' exonuclease
activity inherent in the polymerase cleaves the probe,
25 separating the reporter molecule from the vicinity of the
quencher. The increase in fluorescent reporter signal within a
reaction is a direct measure of the accumulation of PCR
product.

The ABI 7700 has a built in thermal cycler and a laser directed at each sample well in which PCR is performed via bi-directional fibre optic cables. Emitted fluorescence travels through the cables to a detector, where signals from released reporter molecules which fall between 520 and 660nm are collected every few seconds. The increase in fluorescent reporter signal within a reaction is a direct measure of the accumulation of PCR product. The output of the assay is measured as a normalised reporter value, ΔR_n . The normalised reporter value for any sample is calculated by the ABI 7700 sequence detection software by dividing the reporter fluorescent signal by the signal from a reference reporter (ROX) incorporated in the reaction and deducting a baseline fluorescence signal which is established during the first few cycles of PCR. The ΔR_n values are plotted against thermal cycle number to allow visualisation of the extent of PCR product generation. The starting copy number of a target sequence (C_n) is established by determining the fractional cycle number (C_t) at which a PCR product is first detected - the point at which the fluorescence signal passes above a threshold baseline.

To determine C_t the Sequence Detection application collects data from the first few PCR cycles and calculates the average R_n and the standard deviation of the R_n by a default factor of 10 to define a threshold. Finally, the algorithm searches the data for a point that exceeds the baseline by the value of the threshold. The cycle at which this point occurs is defined as C_t . C_t represents a detection threshold for the Sequence Detector. C_t is dependent on the starting template copy number, and the efficiency of both the DNA amplification the PCR system and the cleavage of the fluorogenic probe. When the starting concentration of a template remains constant as the

concentration of other PCR components varies, the most efficient PCR system has the lowest Ct.

Quantification of target is established through comparison of experimental Ct values with a standard curve.

5 The conventional use of the Perkin Elmer TaqMan® assay system had been limited to the use of single dye probes. However, in recent times the system has been adapted for use of multiple reporter dyes within a single reaction tube to perform a convenient multiplex assay in which the mRNA copy number for
10 more than one target gene transcript can be determined within a single tube. To achieve this there must be more than one primer pair present in the tube which presents a problem if there are differences in the initial copy numbers of the gene sequences to be expressed. The more common gene sequence may
15 well exhaust the reagents within the tube preventing amplification of the less common sequence(s).

Perkin Elmer have sought to overcome this problem by limiting the production of the assay PCR products derived from each of the two or more target or marker gene sequences to ensure that
20 if one of the sequences is more abundant than another there will nonetheless be sufficient reagents remaining in the reaction mix to amplify the less abundant even if its amplification and hence detection does not start until the reaction involving the more abundant transcript has run to
25 completion.

In their instructions on how to implement their multiplexing method, Perkin Elmer advise the user (user Bulletin #2 ABI PRISM 7700 Sequence Detection System of December 11 1997) that if the user does not know the relative abundance of each RNA to

be assayed for, then limiting primer concentrations will need to be defined for each by running a matrix of forward and reverse primer concentrations for each and identifying the concentrations as those that show a reduction in ΔR_n but little effect on C_t (threshold cycle). Such characterisation of limiting primer concentrations is inherently time consuming. However, the only alternative proposed by Perkin Elmer is to use rRNA as one of the target gene transcripts avoiding the need for determining limiting primer concentration based upon the fact that rRNA is known to be extremely abundant and will therefore inevitably be more abundant than the other chosen target mRNA in the assay.

It is one general objective of the present invention to provide a method of multiplexing that avoids the need for characterisation of the limiting primer conditions for each of the gene targets without being restricted to use of rRNA as one of the targets.

Summary of the Invention

According to the present invention there is provided a method of quantitative analysis of gene expression utilising a respective fluorescent reporter signal for at least a first and a second target gene within a single replication reaction vessel the method comprising:

(i) determining a suitable restricted concentration of primer for replication of the first target gene, wherein the concentration of primer is selected to provide a robust, but limited fluorescent amplification signal; and

(ii) determining a suitable concentration of primer for

replication of the second target gene, wherein the concentration of primers is selected to give the most efficient detection of amplification as measured by accumulation of fluorescent signal; and

- 5 (iii) utilising the selected primer concentration, determining a suitable concentration of second target gene probe, wherein the concentration of the second target gene probe is selected to provide a fluorescent signal that is at least four times greater than the fluorescent signal of the selected first gene
10 primer concentration.

Suitably the concentration of primer is, in each case, determined for both forwards and reverse primers. As used in step (i) of the method the term "limited" means not maximal and the term robust means strong and readily recognisable.

- 15 Preferably in step (ii) the concentration of primer(s) is selected to give the earliest detection of amplification.

Suitably the first gene primer concentrations correspond to ΔR_n of less than 0.3 and preferably the selected target gene probe concentration corresponds to a target fluorescent signal ΔR_n of
20 between 1.5 and 6.

In a more particular aspect, the invention provides a method of quantitation of expression of a first and a second target protein-encoding nucleic acids using a PCR based assay in which amplification of nucleic acid is detected by release of a
25 reporter fluorescent signal which is measured as a normalised reporter value (" ΔR_n "), the method comprising:

... providing a limiting concentration of a first primer pair, the first primer pair being adapted for amplification of a

desired first target, the limiting concentration being selected to provide a relatively low ΔR_n value as measured by release of a first fluorescent reporter compound from a first reporter probe;

5 selecting a non-limiting concentration of a second primer pair and probe set, the second primer pair being adapted for amplification of a desired second target, the amplification being measured by release of a second fluorescent reporter compound from a second reporter probe, the concentration being
10 selected to provide a ΔR_n value of from three to forty times that provided by said first reporter compound;

 bringing said first and second primer pairs and probes at their limited and selected concentrations respectively into contact with a sample of nucleic acid;

15 performing a polymerase chain reaction under conditions in which the release of the first and second reporter compounds is measured; and

 quantitating the amount of first and second target nucleic acids.

20 Description of Preferred Embodiment.

The general protocol of the method embodying the present invention will now be outlined and then supported by experimental examples.

As indicated above, the invention provides an efficient and
25 time-saving solution for the measurement of two different target nucleic acids in a single sample, without being limited to either selecting rRNA as one of the samples or needing to determine limiting primer concentrations for both targets. In this way, two different protein-encoding nucleic acid targets,
30 particularly mRNA (optionally in the form of cDNA) (although

genomic DNA may also be measured).

In measuring mRNA, a particularly preferred limited reporter probe is one which spans two exon sequences in such a manner that it does not hybridise to either of the two exons individually under PCR conditions but is capable of hybridising to the sequences when brought together in mRNA (for example the probe could be 24 nucleic acids in size representing 12 nucleic acids of the 3' end of the first exon and 12 of the 5' end of the second exon). This provides a level of additional control to ensure the amplified product detected is from a good quality mRNA preparation. Thus the invention may be used to provide quantitation of two nucleic acid targets from genes which are transcribed into polyA⁺ RNA, spliced and translated.

In one embodiment, the first target may be a marker gene such as a ubiquitously expressed marker gene. Some of these genes may sometimes show little or no variation in expression between tissues, and may be used as a means of normalising different samples for loading of starting material whilst measuring the abundance of a second target within them.

Examples of such marker genes include genes involved in basic biosynthetic and metabolic pathways, for example glyceraldehyde-3-phosphate dehydrogenase, alpha actin, heat shock protein 90a, DAD-1 and collagen 3.

Alternatively the first target nucleic acid, as well as the second target nucleic acid, may be transcripts of genes whose expression level varies between different samples, such as different tissue types from the human or animal body. Such targets include oncogenes (e.g. *myc*, *ras*, *erb*, and the like), receptors (e.g. TNF- α , interferon and interleukin subtype

receptors), cytokines (e.g. TNF- α , interferons, interleukins, and the like), growth factors (EGF, PDGF, VEGF, etc), or members of the immunoglobulin superfamily (including T-cell receptors and HLA genes). Targets may also include
5 uncharacterised or partially characterised EST sequences, e.g. EST sequences which include particular domains of interest.

The flanking PCR primer concentrations of the first (e.g. marker) nucleic acid target can be determined in a single experiment by combining a range of forward primer
10 concentrations, typically 10-50nM with an equivalent range of reverse primer concentrations in the presence of an excess concentration of probe (100nM). The concentrations of primers selected should provide a robust, but limited fluorescent amplification signal, typically with a ΔR_n of less than 0.5.
15 preferably less than 0.3, as determined in relation to the ROX baseline presently used in the ABI 7700 system used in the art. This determination will provide a limiting concentration of primers for the first primer pair. The limiting concentration is defined by the ABI User Bulletin #2 as that providing a
20 reduction in the ΔR_n value (compared to that which provides for exhaustion of reagents before primers) but which has little effect on the threshold C_t . In practice, the aim is to determine a concentration such that the ΔR_n value is lowered to, or just above, the value at which the C_t starts to increase
25 when the primer concentrations are lowered further. When measured against the normalised reporter ROX, the ΔR_n for the limiting primer concentration is preferably less than 0.5, more preferably less than 0.3.

It is important that the fluorescent signal generated in this
30 reaction is limited for two reasons. Firstly, the emission spectra of the two dyes currently most often employed in the

reporter probes (e.g. selected from 6-FAM and VIC or JOE) overlap. If a large amount of fluorescence is generated from the first target nucleic acid it can contribute to the signal generated from the second reporter probe fluorophore causing
5 that signal to apparently start to accumulate early and therefore give an inaccurate determination of second target abundance. Secondly, the limited generation of a fluorescent signal (where the fluorescent probe is not the limiting reagent) reflects limited production of first target amplicon,
10 this preserves reagents for amplification of the target of interest.

It is not essential that the determination of the limiting concentration is performed each time a second target gene is quantified.

15 It is also a feature of the invention that unlike the protocol of the prior art, it is not necessary to make a determination of a limiting concentration of the primers and probe set for the second target gene. Instead, we have been able to define a simple and robust set of conditions which, if met, will result
20 in an accurate dual quantitation of two target nucleic acids in a single sample. These conditions are in part a measure of the efficiency of amplification of the second primer and probe set, such that when replicated in a reaction with a limiting concentration of a first primer and probe set, will allow for
25 both target genes to be quantified, irrespective of their relative abundance.

In a typical selection, we provide a known number of copies of a target nucleic acid together with primer and probe concentrations which are non-limiting in the sense that they
30 could be lowered further without significantly altering the Ct.

Typically, we provide about 10,000 copies of a target nucleic acid (optionally in the form of genomic DNA (provided the probe is not exon-spanning)) and at least 250 nM of each of the forward and reverse primers (e.g. from 250 to 500 nM, e.g. about 250-350 nM, most preferably about 300 nM, the concentrations of forward and reverse primers being independently selected but desirably the same), and at least 100 nM of primer probe, (e.g. from 100 to 300 nM, preferably about 150 to 250 nM, e.g. about 200 nm). The term "about" indicates a margin of error of $\pm 10\%$.

A PCR is performed in which amplification of the nucleic acid is detected by release of a reporter fluorescent signal which is measured as a normalised reporter value (" ΔRn ") by reference to the reporter dye ROX. Where the ΔRn is found to be from 1.5 to 6.0 and the Ct is found to be between 24 and 26, then the primer and probe set may be used at the selected concentrations without any need for determination of a limiting concentration. Should the ΔRn and Ct values fall outside the target range, the probe and/or primer concentrations may be adjusted as required in order to achieve the target range.

This range of ΔRn values will thus be from 3 to 40 times the preferred range of ΔRn values for the first target nucleic acid. More preferably, the range is from 4 to 40, even more preferably from 4 to 30 and most preferably from 4 to 20 times.

The conditions here are of course relative to the copy number of the target nucleic acid, and may be adjusted by those of skill in the art to allow for greater or lower copy numbers of second target, with a consequential adjustment in the acceptable Ct .

Once the second primer pair concentration has been selected, the dual quantitation of the first and second target may be performed. The reaction will use standard PCR conditions, and be run typically for 30 - 40 cycles. Suitable conditions may be provided with a variety of commercially available PCR kits, including those designed for use with commercial PCR apparatus, such as the ABI Prism 7700. The conditions set out in the accompanying examples may be used. The concentrations of both deoxynucleotides and polymerase (e.g. AmpliTaq®) enzyme must be sufficient to support amplification of two targets.

The method of the present invention may be used across multiple RNA samples derived from different tissues to generate maps of transcript expression or to profile the expression of many different transcripts in the same RNA sample.

In quantitating the amount of first and second target nucleic acids, this may be by determination of copy number, calculated by reference to the Ct value (this can be performed by the software provided for use with PCR detection apparatus) or may be a relative quantitation, e.g. the ratio of targets in the sample or between different samples (e.g. different tissue types of the human or animal body).

The fluorescent reporter dyes may be any dye which can be attached to a reporter nucleic acid probe and which has a suitable λ_{\max} for detection in a PCR apparatus, generally between 500 and 660 nm. Commercially available dyes include:

- 6-FAM (6-carboxy-fluorescein);
- JOE (2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein);
- ROX (carboxy-X-rhodamine);
- TET (tetrachloro-6-carboxy-fluorescein); and
- VIC (proprietary to and available from Perkin Elmer).

In the invention, it is desirable to use two dyes which have a large difference in emission maxima, such as 6-FAM (λ_{max} 518 nm) and JOE (λ_{max} 554) or 6-FAM and VIC (λ_{max} 550 nm).

5 The reporter dyes are quenched by a suitable quencher, of which many exist, including a variety of rhodamine dyes. A suitable dye is TAMRA (6-carboxy-tetramethyl-rhodamine).

10 The normalised reporter value may be determined against a dye with a λ_{max} above the values of the reporter dyes. A currently preferred reference dye is ROX, against which the values described herein have been normalised. Where a different reference dye is used, the values given herein may be adjusted by recalculation by reference to the difference in values of ROX and the other reference.

15 In summary, the only previously described TaqMan[®] assay incorporating two targets in a single tube requires optimisation of reaction conditions for both marker and target genes for every assay configured unless rRNA is used as the marker gene. The system described here allows a single determination of marker gene conditions which can then be used
20 universally with any target optimised to run in conjunction with it. The system offers a significant advantage over that previously described by allowing choice of marker gene transcript, reducing assay development time and therefore increasing throughput for researchers characterising multiple
25 gene targets. Furthermore the method of the present invention in contrast to the prior art economises on use of the expensive primers, thereby providing valuable cost economy in the execution of the assay.

The present invention is illustrated by the following examples.

Example 1**Primer/probe design.**

A pair of primers and a TaqMan® probes were designed to amplify a specific sequence from a human seven transmembrane receptor
5 (GenBank accession number L24470)

Primer 1:	5'AAGGATCTAAGATTTGATTCCATGTTG3'	SEQ ID NO:1
Primer 3:	5'GAATAAATGGAAATCATTCCTCTGGAAAC3'	SEQ ID NO:2
Probe 5:	5'TGTGAAACAACACTTTTGTCTCTCCGAATG3'	SEQ ID NO:3

10 In addition a pair of primers and a TaqMan® probe were designed which cross an intron/exon boundary and amplify a portion of the human GAPDH gene

Primer 2:	5'CAGAGTTAAAAGCAGCCCTGGT3'	SEQ ID NO:4
Primer 4:	5'GAAGGTGAAGGTCCGAGTCAAC3'	SEQ ID NO:5
Probe 6:	5'TTTGGTCCGTATTGGGCGCCT3'	SEQ ID NO:6

15 Probe 5 is labelled with the fluor 6-FAM while probe 6 is labelled with the fluor JOE.

DNase treatment of total RNA.

For each tissue tested 2.2µg of total RNA was digested with 2 units of Rnase free Dnase (Gibco BRL) for 15 minutes at room
20 temperature in a 20µl volume of 1x Dnase buffer (Gibco BRL). The reaction was stopped by addition of 2µl of 25mM EDTA solution. The samples were then incubated at 65°C for 10 minutes to inactivate the enzyme.

First strand cDNA synthesis.

For each tissue tested 100ng of total RNA was used as template for first strand cDNA synthesis. The RNA in a volume of 4 μ l and in the presence of 50nM primers 1 and 2, 1xPCR buffer II (Perkin Elmer) and 5mM MgCl₂, was heated to 72°C for 5 minutes and cooled slowly to 55°C. After addition of all other reagents, the 6 μ l reaction was incubated at 48°C for 30 minutes followed by an enzyme inactivation step of 90°C for 5 minutes. The final reaction conditions were as follows: 1xPCR buffer II, 5mM MgCl₂, 1mM dATP, dUTP, dGTP, dCTP, 12.5 units MuLV reverse transcriptase (Gibco BRL). By increasing the scale of the reaction it is possible to prime for up to six targets in one reverse transcription.

PCR amplification of first strand cDNA products.

The cDNA derived from 100ng total RNA for each sample was subjected to PCR amplification in a single reaction to identify both target and GAPDH transcripts. The final primer/probe concentrations for target were 300nM primer 1, 300nM primer 3 and 150nM probe 5, those for GAPDH were 20nM primer 2, 20nM primer 4 and 100nM probe 6. The final concentration of other reagents in the reaction were 4.5% glycerol, 1 x 1 TaqMan[®] buffer A (Perkin Elmer), 6.25mM MgCl₂, 430 μ M dATP, dUTP, dGTP, dCTP, 2.5 units AmpliTaq[®] Gold. The PCR amplification was carried out in the ABI 7700 sequence detection system, an initial enzyme activation step of 94°C for 12 min was followed by 45 cycles of 94°C 15 secs, 60°C 1 min (minimum ramp time). For comparative purposes cDNA derived from 100ng cDNA was subjected to PCR amplification in a reaction containing only the primer/probe set for target and run in previously optimised assay conditions of 5% glycerol, 6.5mM MgCl₂, 300 μ M dATP, dUTP, dGTP, dCTP, 1.25 units AmpliTaq[®] Gold.

Results.

The results for five different RNA samples are illustrated in Table 1 and compare both the threshold cycle for the detection of FAM fluorescent signal (Ct) and the log of the starting copy (log Cn) number for the target transcript in the single and dual probe reactions. The correlation coefficient for both groups of data is greater than 0.9 showing that there is almost absolute proportionality in detection of target between the two data sets and that the presence of an additional fluorescent probe in the reaction does not significantly affect the accuracy of specific sequence detection. The threshold cycle for detection of JOE fluorescent signal for GAPDH is shown in table 2 and can either be used as a marker for the presence of intact RNA or as a means of normalisation in an appropriate experimental set up. The average ΔRn in the experimental run for the target primer/probe set is 3.19 and that for the marker gene 0.15.

Table 1

Tissue	Dual probe	Single probe	Dual probe	Single probe
	Ct (6-FAM)	Ct (6-FAM)	Log Cn	Log Cn
uterus endometrium	33.27	33.11	2.27	2.30
liver	27.28	27.34	3.60	3.59
gall bladder	27.48	27.58	3.55	3.53
stomach fundus mucosa	28.10	27.88	3.41	3.46
ileum mucosa	32.69	32.41	2.39	2.46

Table 2

Tissue	Dual probe Ct (JOE)
uterus endometrium	21.48
liver	21.16
gall bladder	21.14

stomach fundus mucosa 23.36
ileum mucosa 24.2

Example 2

Primer probe design.

- 5 A pair of primers and TaqMan® probes were designed to amplify a specific sequence from a human seven transmembrane receptor (GenBank accession number U19487)

Primer 7:	5'TCCGCAGCGGCTTCTC3'	SEQ ID NO:7
Primer 8:	5'GCCTGCAACTTCAGTGTTCATTC3'	SEQ ID NO:8
10 Probe 9:	5'CATCCGCATGCACCGCCG3'	SEQ ID NO:9

These were used in conjunction with the GAPDH primer/probe set described in Example 1.

Probe 9 is labelled with the fluor 6-FAM while probe 6 is labelled with the fluor JOE.

15 DNase treatment of total RNA.

For each tissue tested 2.2µg of total RNA was digested with 2 units of Rnase free Dnase (Gibco BRL) for 15 minutes at room temperature in a 20µl volume of 1xDnase buffer (Gibco BRL). The reaction was stopped by addition of 2µL of 25mM EDTA
20 solution. The samples were then incubated at 65°C for 10 minutes to inactive the enzyme.

First strand cDNA synthesis.

For each tissue tested 100ng was used as template for first strand cDNA synthesis. The RNA in a volume of 4µl and in the

presence of 50nM primers 7 and 2, 1xPCR buffer II (Perkin Elmer) and 5mM MgCl₂ was heated to 72°C for 5 minutes and cooled slowly to 55°C. After addition of all other reagents, the 6μl reaction was incubated at 48°C for 30 minutes followed by an enzyme inactivation step of 90°C for 5 minutes. The final reaction conditions were as follows: 1xPCR buffer II, 5mM MgCl₂, 1mM dATP, dUTP, dGTP, dCTP, 12.5 units MuLV reverse transcriptase (Gibco BRL). As with Example 1, it is possible, by increasing the scale of the reaction, to prime for up to six targets in one reverse transcription.

PCR amplification of first strand cDNA products.

The cDNA derived from 100ng total RNA for each sample was subjected to PCR amplification in a single reaction to identify both target and GAPDH transcripts. The final primer/probe concentrations for target were 300nM primer 7, 300nM primer 8 and 150nM probe 9 (these concentrations gave an Rn of 4.8 in the optimisation protocol), those for GAPDH were 20nM primer 2, 20nM primer 4 and 100nM probe 6. The final concentration of other reagents in the reaction were 4.5% glycerol, 1 x TaqMan® buffer A (Perkin Elmer), 6.24mM MgCl₂, 430μM dATP, dUTP, dGTP, dCTP, 2.5 units AmpliTaq® Gold. The PCR amplification was carried out in the ABI 7700 sequence detection system, an initial enzyme activation step of 94°C for 12 min was followed by 45 cycles of 94°C 15 secs, 60°C 1 min (minimum ramp time). For comparative purposes cDNA derived from 100ng cDNA was subjected to PCR amplification in a reaction containing only the primer/probe set for target and run in previously optimised assay conditions of 5% glycerol, 6.5mM MgCl₂, 300μM, dATP, dUTP, dGTP, dCTP, 1.25 units AmpliTaq® Gold.

30 Results.

The results of five different RNA samples are illustrated in Table 3 and compare both the threshold cycle for the detection of 6-FAM fluorescent signal (Ct) and the log of the starting copy (log Cn) number for the target transcript in the single and dual probe reactions. The correlation coefficient for both groups of data is greater than 0.9 showing that there is almost absolute proportionality in detection of target between the two data sets and that the presence of an additional fluorescent probe in the reaction does not significantly affect the accuracy of specific sequence detection. The threshold cycle for detection of JOE fluorescent signal for GAPDH is shown in Table 4 and can either be used as a marker for the presence of intact RNA or as a means of normalisation in an appropriate experimental set up. The average ΔRn in the experimental run for the target primer/probe set is 3.85, that for the marker gene 0.14.

Table 3

Tissue	Dual probe Ct (6-FAM)	Single probe Ct (6-FAM)	Dual probe Log Cn	Single probe Log Cn
uterus endometrium	30.3	30.5	2.81	2.77
liver	30.39	30.03	2.81	2.89
gall bladder	29.3	29.44	3.07	3.04
stomach fundus mucosa	30.1	29.4	2.88	3.04
ileum mucosa	32.4	32.06	2.34	2.41

Table 4

Tissue	Dual probe Ct (JOE)
uterus endometrium	20.46
liver	20.06
gall bladder	20.84
stomach fundus mucosa	23.02

ileum mucosa

23.26

Example 3.

Pairs of primers and TaqMan® probes were designed to amplify specific sequences for the following targets (listed as GenBank
5 accession numbers):

X52773	D11131	U03504	U87460
U66661	J05556	M63904	X15376
X57766	AF043101		

These were used in conjunction with the GAPDH primer/probe set
10 described in Example 1 to amplify their respective targets from a total RNA sample isolated from human liver. The probes for the targets listed above were all labelled with the fluor 6-FAM while the probe for GAPDH is labelled with JOE.

15 Selection of primer/probe concentrations for the non limited targets.

A PCR reaction is performed using the target gene primers and probe at a final concentration of 300nM forward primer, 300nM reverse primer and 200nM probe on 30ng of human genomic DNA in a 25µl reaction containing 4.5% glycerol, 1xTaqMan® buffer A
20 (Perkin Elmer) 6.25mM MgCl₂, 430µM dATP, dUTP, dGTP, dCTP, 2.5 units of AmpliTaq® Gold. The PCR amplification was carried out in the ABI7700 sequence detection system, an initial enzyme activation step of 94°C for 12 minutes was followed by 40 cycles of 94°C 15 seconds, 60°C 1 minute (minimum ramp time).
25 If the reaction fulfils the following criteria, the primer/probe set can be used in conjunction with any primer limited set irrespective of the relative abundance of the two

targets.

- The Ct falls between 24 and 26 (where the threshold is set at 0.15)
- ΔRn at reaction plateau falls between 1.5 and 6
- 5 • A single reaction product of the predicted size is generated by the PCR reaction as assessed by gel electrophoresis
- If the ΔRn is >6 or <1.5 the probe concentration can be adjusted without further experimentation to fulfil the
- 10 reaction parameters by assuming a linear relationship between probe concentration and ΔRn .

Where the ΔRn was outside the above range, the selection protocol was re-run with 50nM more or less probe so that the ΔRn value was increased or decreased as required to meet the

15 criteria.

The following table shows the Ct and ΔRn values for the non-limiting forward (F) and reverse (R) primer and probe (P) concentrations selected.

Target	Concentration F/R/P	Ct on 30ng of human genomic DNA	ΔRn
20 X52773	300/300/150	24.02	4
D11131	300/300/150	24.16	6
U87460	300/300/250	24.03	4
U03504	300/300/250	24.744	1.6
U66661	300/300/250	24.399	3.5
25 J05556	300/300/250	24.58	4
M63904	300/300/150	24.346	4.5
X15376	300/300/200	24.41	3

X57766	300/300/250	25.855	5
AF043101	300/300/200	24.46	3

The selected probe-primer set was used in multiplex assay as follows.

5 DNase treatment of total RNA.

2.2 μ g of total RNA isolated from human liver was digested with 2 units of RNase free DNase (Gibco BRL) for 15 minutes at room temperature in a 20 μ l volume of 1xDNase buffer (Gibco BRL).

10 The reaction was stopped by the addition of 2 μ l of 25mM EDTA solution. The samples were then incubated at 65°C for 10 minutes to inactivate the enzyme.

First strand cDNA synthesis.

15 For each transcript to be measured 100ng of total RNA was used as a template for cDNA synthesis. The RNA in a volume of 4 μ l and in the presence of 50nM reverse primers for target and GAPDH, 1 x PCR Buffer II (Perkin Elmer) and 5mM MgCl₂ was heated to 72°C for 5 minutes and cooled slowly to 55°C. After addition of all other reagents, the 6 μ l reaction was incubated at 37°C for 30 minutes followed by an enzyme inactivation step
20 of 90°C for 5 minutes. The final reaction conditions were as follows: 1 X PCR buffer II, 5mM MgCl₂, 1mM dATP, dTTP, dGTP, dCTP, 12.5 units MuLV reverse transcriptase (Gibco BRL).

PCR amplification of first strand cDNA products.

25 The cDNA derived from 100ng total RNA from human liver was subjected to PCR amplification in a single reaction to identify both target and GAPDH transcripts. The final primer/probe

concentrations for target were as shown in the table above, those for GAPDH were 20nM primer 2, 20nM primer 4 and 100nM probe 6. The final concentration of other reagents in the reaction were 4% glycerol, 0.66 x TaqMan® buffer A (Perkin Elmer), 6mM MgCl₂, 358µM dATP, dUTP, dGTP, dCTP, 2.5 units AmpliTaq® Gold. The PCR amplification was carried out in the ABI 7700 sequence detection system, an initial enzyme activation step of 94°C for 12 min was followed by 45 cycles of 94°C 15 secs, 60°C 1 min (minimum ramp time).

10 Results.

The results for the detection of the eleven targets each in 100ng total RNA isolated from human liver are illustrated in the following table and show both the threshold cycle for the detection of 6-FAM and JOE fluorescent signals (6-FAM Ct and JOE Ct) and the starting copy number (Cn) for both the GAPDH and target transcripts in each reaction. The standard error of the mean for the GAPDH Ct values is 0.011621 showing that the reaction proceeds with the same efficiency irrespective of the abundance of the second target.

Target	6-FAM Ct	6-FAM Cn	JOE Ct	JOE Cn
X52773	21.76	108,751	22.04	91,023
D11131	23.61	33,559	21.97	95,164
U87460	26.04	7,163	22.07	89,304
U03504	28.95	1,127	22.07	89,304
U66661	29.96	593	22.03	91,803
J05556	31.68	199	22.02	92,187
M63904	32.52	117	22.12	86,510
X15376	36.8	8	22.04	91,023
X57766	37.09	6	22.07	89,304
AF043101	39.52	1	22.06	89,873

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity and understanding, it will be readily apparent to those of skill in the art that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A method of quantitation of expression of a first and a second target protein-encoding nucleic acids using a PCR based assay in which amplification of nucleic acid is detected by release of a reporter fluorescent signal which is measured as a normalised reporter value (" ΔRn "), the method comprising:

providing a limiting concentration of a first primer pair, the first primer pair being adapted for amplification of a desired first target, the limiting concentration being selected to provide a relatively low ΔRn value as measured by release of a first fluorescent reporter compound from a first reporter probe;

selecting a non-limiting concentration of a second primer pair and probe set, the second primer pair being adapted for amplification of a desired second target, the amplification being measured by release of a second fluorescent reporter compound from a second reporter probe, the concentration being selected to provide a ΔRn value of from three to forty times that provided by said first reporter compound;

bringing said first and second primer pairs and probes at their limited and selected concentrations respectively into contact with a sample of nucleic acid;

performing a polymerase chain reaction under conditions in which the release of the first and second reporter compounds is measured; and

quantitating the amount of first and second target nucleic acids.

2. A method according to claim 1 wherein said reporter value is normalised by reference to the reporter dye ROX, and said relatively low ΔRn value is less than 0.5.

3. A method according to claim 2 wherein the ΔR_n of said second reporter compound is from 1.5 to 6.0.

4. A method according to any one of the preceding claims wherein said selecting is by a process which comprises:

providing about 10,000 copies of said second target nucleic acid;

providing primer concentrations in excess of 250 nm and a probe concentration in excess of 100 nm;

performing an amplification reaction in which amplification of nucleic acid is detected by release of a reporter fluorescent signal which is measured as a normalised reporter value (" ΔR_n "),

selecting the primer and probe concentrations used as a non-limiting concentration when the fractional cycle number at which amplification of a second target sequence is detected is from 24 to 26 and the reporter value is normalised by reference to the reporter dye ROX is from 1.5 to 6.0.

5. A method according to any one of the preceding claims wherein said first desired target is a ubiquitously expressed marker gene.

6. A method according to claim 5 wherein said marker gene is GAPDH.

SEQUENCE LISTING

<110> Pharmagene Laboratories Ltd.
Carey, Jane Elizabeth

<120> Quantitative Analysis of Gene Expression

<130> AHB/CP5787510

<140>

<141> 1999-07-21

<150> GB 9815799

<151> 1998-07-21

<160> 9

<170> PatentIn Ver. 2.1

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 1

aaggatctaa gatttgattc catgttg

27

<210> 2

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 2

gaataaatgg aaatcattct ctggaaac

28

<210> 3

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 3

tgtgaaacaa cactttttgc tctccgaatg

30

<210> 4

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 4

cagagttaaa agcagccctg gt

22

<210> 5

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 5

gaaggtgaag gtcggagtca ac

22

<210> 6

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 6

tttggtccgt attgggcgcc t

21

<210> 7

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 7

tccgcagcgg cttctc

16

<210> 8

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 8

gcctgcaact tcagtgtcat tc

22

<210> 9

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 9

catccgcatg caccgccg

19

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/02359

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WOUDENBERG T M ET AL: "QUANTITATIVE PCR BY REAL TIME DETECTION" PROCEEDINGS OF THE SPIE, XP000197422 page 312 -page 314 ---	1-6
A	HEID C A ET AL: "REAL TIME QUANTITATIVE PCR" GENOME RESEARCH, US, COLD SPRING HARBOR LABORATORY PRESS, vol. 6, no. 10, page 986-994 XP000642795 ISSN: 1088-9051 the whole document ---	1-6
A	WO 97 29210 A (PERKIN ELMER CORP) 14 August 1997 (1997-08-14) claims 1-13 --- -/--	1-6

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 November 1999

Date of mailing of the international search report

17/11/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/02359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 46714 A (RASMUSSEN RANDY P ;UNIV UTAH RES FOUND (US); RIRIE KIRK M (US); WI) 11 December 1997 (1997-12-11) page 2, paragraph 2 -page 3, paragraph 2 page 5, paragraph 11 -page 10, paragraph 6; claims 15-34 -----	1-6
A	EP 0 713 921 A (HOFFMANN LA ROCHE) 29 May 1996 (1996-05-29) claims 1-3 -----	1-6
A	EP 0 639 647 A (TANABE SEIYAKU CO ;EIKEN CHEMICAL (JP)) 22 February 1995 (1995-02-22) the whole document -----	1-6
A	WO 94 23023 A (UNIV ROCHESTER) 13 October 1994 (1994-10-13) claims 1,2 -----	1,5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

Pct/GB 99/02359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729210 A	14-08-1997	US 5716784 A	10-02-1998
		AU 700521 B	07-01-1999
		AU 1856697 A	28-08-1997
		CA 2243759 A	14-08-1997
		EP 0870063 A	14-10-1998
		JP 11506020 T	02-06-1999
WO 9746714 A	11-12-1997	AU 3154797 A	05-01-1998
		AU 3380097 A	05-01-1998
		AU 3481297 A	05-01-1998
		CA 2256773 A	11-12-1997
		CA 2257109 A	11-12-1997
		EP 0912760 A	06-05-1999
		EP 0906449 A	07-04-1999
		EP 0912766 A	06-05-1999
		WO 9746707 A	11-12-1997
		WO 9746712 A	11-12-1997
EP 0713921 A	29-05-1996	US 5571673 A	05-11-1996
		AU 700885 B	14-01-1999
		AU 3901095 A	30-05-1996
		CA 2163388 A	24-05-1996
		CN 1133888 A	23-10-1996
		JP 8220092 A	30-08-1996
		NZ 280506 A	27-07-1997
EP 0639647 A	22-02-1995	JP 7023800 A	27-01-1995
WO 9423023 A	13-10-1994	US 5643765 A	01-07-1997
		US 5639606 A	17-06-1997
		AU 6493194 A	24-10-1994
		JP 7132100 A	23-05-1997
		US 5876978 A	02-03-1999